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1 **Hydrogen Production in Photosynthetic Microbial Mats in the Elkhorn Slough**
2 **Estuary, Monterey Bay**

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13 Running Title: H₂ production in photosynthetic microbial mats

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Abstract

Hydrogen (H₂) release from photosynthetic microbial mats has contributed to the chemical evolution of Earth and could potentially be a source of renewable H₂ in the future. However, the taxonomy of H₂-producing microorganisms (hydrogenogens) in these mats have not been previously determined. With combined biogeochemical and molecular studies of microbial mats collected from Elkhorn Slough, Monterey Bay, California we characterized the mechanisms of H₂ production and identified a dominant hydrogenogen. Net production of H₂ was observed within the upper photosynthetic layer (0-2 mm) of the mats under dark, anoxic conditions. Pyrosequencing of rRNA gene libraries generated from this layer demonstrated the presence of 64 phyla, with *Bacterioidetes*, *Cyanobacteria* and *Proteobacteria* dominating the sequences. Sequencing of rRNA transcripts obtained from this layer demonstrated that *Cyanobacteria* dominated rRNA transcript pyrotag libraries. An OTU affiliated to *Microcoleus* spp. was the most abundant OTU in both rRNA gene and transcript libraries. Depriving mats of sunlight resulted in an order of magnitude decrease in subsequent nighttime H₂ production, suggesting that newly fixed carbon is critical to H₂ production. Suppression of nitrogen (N₂)-fixation in the mats did not suppress H₂ production, which indicates that co-metabolic production of H₂ during N₂-fixation is not an important contributor to H₂ production. Concomitant production of organic acids is consistent with fermentation of recently produced photosynthate as the dominant mode of H₂ production. Analysis of rRNA % transcript:% gene ratios and H₂-evolving bidirectional [NiFe] hydrogenase % transcript:% gene ratios indicated that *Microcoelus* spp. are dominant hydrogenogens in the Elkhorn Slough mats.

41 Fermentation/ Hydrogen/Bidirectional [NiFe] hydrogenase/ *Microcoleus* spp./ Pyrotags

Introduction

Microbial mats are among the most phylogenetically and physiologically diverse ecosystems on Earth (Bolhuis and Stal, 2011; Kunin *et al.*, 2008; Ley *et al.*, 2006). These systems undergo daily cycling of redox potential, increasing as oxygenic phototrophs supersaturate the upper strata with oxygen during the day and then decreasing as the mats become completely anoxic at night. While the role of mats in global carbon and oxygen cycling is acknowledged and well studied (Des Marais, 2003; Nisbet and Fowler, 1999), their H₂ ecology has been underexplored.

H₂ release from microbial mats has likely been important for the chemical evolution of Earth, where its emission over long periods of geological time might be an important contributing factor to the oxidation of our atmosphere (Hoehler *et al.*, 2001). Today, H₂ is an important industrial chemical, where 10⁸ m³ (at 1 atm) are sold annually in the US alone (Lee *et al.*, 2010). Biological systems capable of producing industrially significant amounts of H₂ have been an important focus of alternative energy research. Mat ecosystems could thus play a potential role as a renewable source of H₂ in the future (Bender and Phillips, 2004). Knowledge of the metabolic processes that lead to H₂ generation and the phylogenetic identity of associated H₂-producing microorganisms (hydrogenogens) within highly complex microbial mat communities will be an important initial step towards realizing the potential of these systems as a viable H₂ source. This knowledge will be useful in optimizing system parameters, such as light, carbon and nutrient concentrations that enhance hydrogenogen activity and may facilitate future use of microbial mats as a commercial source of H₂.

The activity of phototrophs and diazotrophs is well established in photosynthetic microbial mats (Des Marais, 2003) and both groups are capable of producing H₂, either fermentatively or as a co-metabolic product of N₂-fixation (Bothe *et al.*, 2010). Recent molecular ecology studies of hydrogenogens in microbial mats have focused on use of the H₂-evolving bidirectional [FeFe] hydrogenase as a biomarker for investigating hydrogenogens in photosynthetic mats (Boyd *et al.*, 2009; Boyd *et al.*, 2010). These studies infer that non-phototrophic, anaerobes are important hydrogenogens in mat ecosystems. However, the class of hydrogenases targeted and the assays used in these studies did not include H₂-evolving bidirectional [NiFe] hydrogenases harbored by phototrophic microorganisms. To our knowledge no studies have examined the diversity of H₂-evolving bidirectional [NiFe] hydrogenases in microbial mats that may be active in phototrophic hydrogenogens that produce H₂ by fermentative processes. Thus our current understanding of the overall microbial ecology of H₂ cycling in microbial mats remains an incomplete picture.

Previous work, which employed biogeochemical or molecular approaches individually, has served to constrain several aspects of H₂ cycling in microbial mats (Hoehler *et al.*, 2001; Skyring *et al.*, 1988; Skyring *et al.*, 1989). However, robust linkages between microorganisms and processes that generate H₂ remain to be established and the taxonomic identity of principal hydrogenogens is unknown. In this study we determined the location of hydrogenogens in Elkhorn Slough mats, the relative importance of co-metabolic production of H₂ during N₂-fixation vs fermentation and phototrophic vs. heterotrophic H₂ production. In parallel we used pyrosequencing of SSU rRNA genes

and transcripts and sequencing of H₂-evolving bidirectional [NiFe] hydrogenase genes and transcripts to obtain evidence for the taxonomic identity of dominant hydrogenogens.

Methods

Field site sampling and short-term maintenance of mats in a greenhouse

Photosynthetic microbial mats were sampled from a tidal zone in the Elkhorn Slough estuary at Monterey Bay, California (Latitude 36.830 °N, Longitude -121.785 °W) on November 9, 2009. The samples measured 18 x 23 cm² and were ~2 cm in depth. The samples were transported to the NASA Ames Research Center greenhouse within 1-2 hr of collection from the slough. Upon arrival, mats were placed in acrylic aquaria transparent to ultraviolet radiation and covered with 4 L of water (~3 cm of overlying water column) from the field site. The salinity of the field site water was 35 ‰ and had a pH of 8.0. Mats were incubated under natural solar irradiance and a regulated temperature (~19°C, *in situ* average) for up to 5 days, during which diel cycle experiments were carried out.

Analysis of H₂ and organic acids

Replicate vials were prepared identically for each control or manipulation experiment as follows: small sub-cores (11 mm diameter, 15 mm depth or horizontally sectioned for depth profile analyses) were cut from whole sections of intact microbial mat and placed in serum vials with 4 mL of field site water. Serum vials were capped with butyl rubber stoppers. The 10.5 mL headspace of the serum vials was left as air for light/daytime incubations and was thoroughly flushed with N₂ (gas and liquid phase degassed) for

dark/nighttime incubations. For H₂ analysis, at least six replicate vials were sampled at each time point for each control or manipulation experiment. Organic acid production was analyzed in each of three replicates for each time point for each control or manipulation experiment. Manipulations included sectioning of microbial mat to identify the location of H₂ production and suppression of diazotroph or phototroph (oxygenic and/or anoxygenic) activity to assess the roles of these microbial groups in H₂ production. See supplementary methods for further information regarding H₂ and organic acid analyses and details on how manipulation experiments were performed.

Analysis of N₂-fixation

N₂-fixation activity was determined using the acetylene reduction (ethylene production) assay in triplicate incubations for each time point for each control or manipulation experiment using standard methods (Bebout *et al.*, 1993; Stewart *et al.*, 1967).

Statistical analysis

To determine significant differences between H₂ or organic acid production under control vs. manipulated conditions, a one-way ANOVA analysis was performed using the Tukey-HSD post hoc comparison within the JMP software package (v9; <http://www.jmp.com/software/jmp9/>). If a P-value of < 0.05 was calculated between control and manipulated conditions, the difference was considered significant.

Nucleic acid isolation and cDNA synthesis

Microbial mat nucleic acids were isolated from at least three pooled mat cores, which were incubated in the same way as those analyzed for H₂ and organic acid production.

Mat cores were sampled periodically from serum vials and immediately transferred to 2 ml tubes, flash frozen in liquid nitrogen and stored at -80°C. DNA and RNA were co-isolated from the upper photosynthetic layer (0-2 mm) of mat. DNA-free RNA was reverse transcribed to cDNA using random hexamers and a Superscript III first-strand synthesis kit (Invitrogen, CA, USA). Further information regarding nucleic acid isolation, RNA purification and cDNA synthesis can be found in the supplementary methods.

Pyrosequencing of SSU rDNA and rRNA

The V8 hypervariable region of the SSU rRNA gene was amplified from DNA (rRNA gene) or cDNA (rRNA transcript) templates using the universal primer pair 926f/1392r, including the titanium adaptor sequences and a five-base barcode on the reverse primer according to the previously published protocols for pyrosequencing (Engelbrektson *et al.*, 2010). Amplicons were quality checked, trimmed to a 200-bp sequence length and clustered (97% similarity) using PyroTagger (Kunin and Hugenholtz, 2010). Representative sequences from each cluster (OTU) were compared to the Greengenes NAST-aligned database (DeSantis *et al.*, 2006) or the SILVA database (Pruesse *et al.*, 2007) using PyroTagger (Kunin and Hugenholtz, 2010). Taxonomic affiliations of OTUs were further verified by querying the NCBI non-redundant nucleotide sequence databases (Altschul *et al.*, 1990). If the best match was 100% identical over 200-bp, without multiple best matches to phylogenetically different taxa, then the OTU was conservatively assigned that taxonomic affiliation at the level of genus. If the OTU was <100% but ≥97% identical its taxonomic assignment was conservatively set to the level of order. OTUs with <97% identity matches were set to the level of phylum assigned by

PyroTagger or if not assigned an identity by PyroTagger the OTU was unclassified (Kunin and Hugenholtz, 2010). Richness (Chao1 and ACE) and diversity (Simpson and Shannon) indices were calculated with the vegan package (v1.17-8) for R (v2.12; <http://cran.r-project.org/web/packages/vegan/index.html>). Hughes *et al.* (2001) and Hill *et al.* (2003) provide further information and critical review of these indices application in microbial ecology.

Design of degenerate bidirectional [NiFe] hydrogenase-specific primers, PCR amplification, cloning and sequencing

A database of bidirectional [NiFe] hydrogenase gene sequences from a range of *Bacteria* and *Archaea* was compiled from NCBI non-redundant nucleotide sequence database (Altschul *et al.*, 1990) and the Integrated Microbial Genomes (IMG) database (Markowitz *et al.*, 2006). Gene sequences were converted to protein sequences to screen for the presence of bidirectional [NiFe] hydrogenase signature motifs L1 Ex[APV]xxxxRxCG[IL]Cxx[AS]Hx[IL][ACS][AGS][AGNSV][KR][ATV]xD and L2 DPC[IL]SC[AS][AST]H[ASTV]x[AG]xx[APV] (PROSITE format; (Vignais and Billoud, 2007). Sequences with >2 mismatches to signature motifs were not included in the database. The screened bidirectional [NiFe] hydrogenase gene sequences were aligned using MUSCLE (Edgar, 2004) and the resulting alignments checked manually in Geneious (v5.3, <http://www.geneious.com>). The database of aligned bidirectional [NiFe] hydrogenase gene sequences was queried against candidate degenerate primers using PRIMROSE to determine the number of mismatches between primers and templates and direct selection of the optimal degenerate primer pair (Ashelford *et al.*, 2002). The optimal degenerate primer pair that targeted the broadest diversity of *Cyanobacteria* and

Chloroflexales corresponded to positions 13 to 19 (IEGHAKI) and positions 167 to 173 (WAVPPGGV) in the *M. chthonoplastes* PCC7420 bidirectional [NiFe] hydrogenase protein sequence. Genomic DNA (gDNA) or cDNA was used as the template to amplify a ~480 bp fragment of the bidirectional [NiFe] hydrogenase gene using the degenerate primers HoxH_F37 (5'-ATHGARGGHCA YGCBAARAT-3') and HoxH_R518 (5'-ACNCCICCVGGNAYHGHCCA-3') using the following PCR cycling conditions; 1x 5 min, 95°C (enzyme activation); 35x 1 min, 95°C (denature); 35x 1 min, 56.5°C (anneal); 35x 1 min, 72°C (extend) and 1x 7 min, 72°C (final extension). The 25 µL PCR reaction volume contained 12.5 µL GoTaq green master mix (contains 1.5 mM MgCl₂ and Taq enzyme; Promega), 8 µM of each primer (HoxH_F37/HoxH_R518), 20 µg bovine serum albumin and 1 µL template (10 ng gDNA or 1 µL of cDNA synthesis reaction). Positive and negative controls were also tested in PCR reactions (*M. chthonoplastes* PCC 7420 and *Crocospaera watsonii* WH 8501 respectively) to evaluate the assay for efficiency and specificity. PCR products of the predicted size were excised from agarose gels after electrophoresis and purified using the Wizard SV gel and PCR clean-up system (Promega, WI, USA). Purified PCR product was ligated into the pCR2.1-topo vector (Invitrogen, CA, USA) and subsequently Sanger sequenced on a 3730xl DNA analyzer (Applied Biosystems, CA, USA).

Phylogenetic analysis of bidirectional [NiFe] hydrogenase clone sequences

Clone sequences were quality filtered, trimmed, translated to protein sequences, screened for L1 signature motif regions and aligned to a custom database of bidirectional [NiFe]

hydrogenase protein sequences in Geneious (v5.3, <http://www.geneious.com>). Inferred hydrogenase amino acid sequences were clustered at 97% similarity using CD-HIT (Huang *et al.*, 2010) and representative sequences were queried against the NCBI non-redundant peptide sequence databases (Altschul *et al.*, 1990) to identify closest protein matches for phylogenetic analysis and tree building. Singletons were excluded from further analysis. Phylogenies were inferred using maximum-parsimony (PROTPARS; 1000 bootstraps) and maximum-likelihood (RAxML; PROTMIX rate distribution model; Dayhoff and WAG amino acid substitution models; 1000 bootstraps) evolutionary models in ARB (Ludwig *et al.*, 2004). Tree topologies from the different analyses were similar and the presented tree is based on a maximum-likelihood analysis, with bootstrap support of branch nodes indicated only when supported by all models. Representative clone sequences were deposited in GenBank (Accession numbers JF816258 – JF816271).

Results

H₂ production

A time-course incubation of horizontally sectioned mat was conducted to assess net H₂ production in space and time. H₂ production was observed predominantly at night under anoxic conditions within the upper photosynthetic layer (0-2 mm) of the mat and was an order of magnitude greater than that produced in lower layers (Figure 1A). H₂ produced in intact mat cores (0-15 mm) was similar to H₂ produced in the sectioned mat cores (0 – 2 mm; upper phototrophic layer) when calculated per surface area (exposed top side surface only; Figure S1). This data further supported the observation that H₂ production is principally occurring within the upper layer.

Patterns of H₂ and organic acid production observed in control and manipulation experiments reported here (Figure 1) were reproducible in mats sampled at other times during December-January 2008/2009 and August-October of 2009 (data not shown). However, the magnitude of H₂ and organic acid production sometimes varied between diel cycle studies. This may have been due to community structure heterogeneity between different cores sampled from microbial mat pieces at different times or it may have been due to variable physiological states of hydrogenogens in the system.

H₂ production under phototroph suppressed conditions

Manipulations of photosynthetic activity were performed to assess the role of different photosynthetic pathways and organisms in H₂ production as explained below. In one set of experiments anoxygenic and oxygenic photosynthetic activity facilitated by photosystem (PSI) and photosystem II (PSII) respectively, was suppressed by depriving the mat of all light and thereby imposing continuously dark conditions over a complete diel cycle. In a second set of experiments performed under normal light conditions, DCMU was added to inhibit oxygenic photosynthesis only, a well-known inhibitor of PSII. Inhibition of PSII was confirmed using PAM fluorometry (Table S1) and was further supported anecdotally by the absence of oxygen bubbles, which normally appear at the surface of the mat (Figure S2). Light deprivation and the subsequent cessation of all photosynthetic activity during the day reduced nighttime H₂ production on the following night by an order of magnitude. However, specific and complete inhibition of oxygenic phototrophy (via addition of DCMU; second set of experiments) did not

significantly affect H_2 production under dark, anoxic conditions (Figure 1B). Together these results indicate that prior-day activity of photosynthetic microorganisms is the source of reducing equivalents for H_2 production and further suggest that anoxygenic phototrophy (PSI activity) is fully capable of serving this function in Elkhorn Slough mats. However, these data alone do not distinguish between H_2 production occurring predominantly by (i) obligate oxygenic phototrophs that are replaced by anoxygenic phototrophs under the conditions of our experimental manipulation, (ii) oxygenic phototrophs capable of facultative anoxygenic phototrophy, (iii) anoxygenic phototrophs; or (iv) a combination of (i)-(iii).

Production of H_2 and organic acids under N_2 -fixation suppressed conditions

To evaluate the relative importance of co-metabolic production of H_2 during N_2 -fixation vs. fermentative H_2 production, both of which are consistent with the observed pattern of H_2 production in space and time, we suppressed N_2 -fixation by addition of a combined nitrogen source (NH_4Cl) and quantified the production of H_2 and organic acids (expected products of fermentation). Nighttime N_2 -fixation was completely suppressed when mats were incubated in the presence of NH_4Cl (Figure S3), but H_2 production was not suppressed (Figure 1C). At two latter nighttime time points (3:30 and 6:30), H_2 production actually increased, suggesting that excess NH_4Cl can stimulate H_2 production. During the same nighttime period acetate, formate, and propionate were produced with H_2 under dark, anoxic conditions (Figure 1D). Lactate, butyrate and valerate were not detected. Organic acid accumulation was not significantly different when N_2 -fixation was suppressed under the same conditions (Figure 1D), with the exception of one time point (0:00). Together these results indicate that N_2 -fixation is a relatively insignificant

contributor to H₂ formation and suggest that fermentation is the dominant mechanism of production.

SSU rRNA genes and transcripts

To profile the identity and activity of microorganisms in Elkhorn Slough microbial mats, we compared rRNA gene and transcript pyrosequence tags from four different time points, including day and night samples (Table 1). For each time point DNA and RNA templates were co-isolated from the upper photosynthetic layer (0-2 mm), as our biogeochemical data demonstrated that H₂ was predominantly produced within this layer.

Richness estimates calculated using Chao1 and ACE indices suggested that we had not exhaustively sequenced the upper photosynthetic layer of the microbial mat community (Table 2). Microbial diversity was consistently estimated to be highest in the rRNA gene libraries as determined using either the Simpson (inverse) or Shannon indices, with each rRNA gene library value higher than the value for the corresponding rRNA transcript library (Table 2).

Analysis of all four rRNA gene libraries (n = 17 047 sequences) revealed that 64 phyla/divisions across all three domains of life were present in the upper photosynthetic layer of Elkhorn Slough microbial mats (Table S2). Abundant phyla/divisions included, *Bacteroidetes* (29%), *Cyanobacteria* (22%) and *Proteobacteria* (14%) (average of four libraries; Figure 2). A lower diversity of phyla/divisions were recovered from the upper photosynthetic layer of all four rRNA transcript libraries (n = 19 982 sequences), with 49 phyla/divisions represented (Table S3). *Cyanobacteria* dominated these libraries, representing 71% of all reads (average of four libraries; Figure 2). The rRNA transcript

libraries were dominated by *Microcoleus* spp. affiliated OTU 2 (Library #2, Day 1, 37%; Library #4, Night 1, 47%; Library #6, Day 2, 48%; Library #8, Night 2, 46%; Figure 2) and *Cyanobacteria* affiliated OTU 9 (Library #2, Day 1, 22%; Library #4, Night 1, 18%; Library #6, Day 2, 27%; Library #8, Night 2, 20%; Figure 2). These OTUs are likely important members of the active microbial mat community during the day and also at night.

Relative activity of microbial populations was inferred at the transcript level from the ratio of the fraction of tags in the rRNA transcript library (active microorganisms) to those in the paired rRNA gene library (active + inactive microorganisms) for a given time point. OTUs were considered relatively active if the % rRNA transcripts recovered was greater than the % rRNA genes recovered ie. % rRNA transcripts:% rRNA genes ratio >1 (Jones and Lennon, 2010; Rodriguez-Blanco *et al.*, 2009). These ratios may give additional insight into active microorganisms beyond those that we previously identified from our rRNA transcript libraries (the ‘abundant and active’ OTUs 2 and 9) and identify those organisms that are relatively ‘rare and active’ that are not obvious from examination of rRNA transcript libraries alone. We were specifically interested in detecting microorganisms correlated with H₂ production and thus we report in Table 3 only those OTUs that were most relatively active at night and present in all our nighttime libraries. *Microcoleus* spp. (OTU 2) had the highest nighttime % transcript:% gene ratios of all OTUs analyzed (present in all libraries), 6.0 (Nov/11/2009) and 4.2 (Nov/13/2009). An OTU (225) with the second highest nighttime ratios was affiliated with the *Desulfobacterales*. Bacteria within this order are exclusively sulfate-reducing bacteria (SRB) and these microorganisms may also be H₂-consumers (hydrogenotrophs; Widell,

1987). The most relatively active nighttime OTUs present in all nighttime libraries were predominantly microbial groups within either the *Cyanobacteria* or *Chloroflexi*. This data correlates with our biogeochemical data and generally supports the hypothesis that *Cyanobacteria*, including *Microcoleus* spp. (oxygenic phototrophs) and/or members of the *Chloroflexi* (anoxygenic phototrophs) are hydrogenogens in Elkhorn Slough mats.

H₂-evolving bidirectional [NiFe] hydrogenase genes and transcripts

H₂-evolving bidirectional [NiFe] hydrogenases are found in a diversity of *Cyanobacteria*, *Chloroflexi* and *Proteobacteria* (also other *Bacteria* and *Archaea*) and are the only known hydrogenases in these taxonomic groups capable of evolving H₂ via fermentation (Tamagnini *et al.*, 2007; Vignais and Billoud, 2007). Thus genes encoding for these enzymes represent useful biomarkers for evaluating the diversity of hydrogenogens in photosynthetic microbial mats. [NiFe] hydrogenases are quite diverse (Vignais and Billoud, 2007) and therefore it is impossible to design degenerate primers that capture the entire diversity that may be present in highly complex ecosystems such as microbial mats. However, as our biogeochemical and pyrosequencing data indicated that the important hydrogenogens in this system are potentially members of the *Cyanobacteria* and *Chloroflexales*, we focused on amplifying bidirectional [NiFe] hydrogenases from these two groups, for which it was possible to design a single PCR-based assay.

Amplicons were generated that spanned the L1 signature motif region of the large subunit of the bidirectional [NiFe] hydrogenase. The primer pair HoxH_F37/HoxH_R518 designed in this study generated an amplicon of ~480 bp, shorter than the bidirectional [NiFe] hydrogenase-specific primer pair HoxHf/HoxHr that produces a ~1190 bp

amplicon (Barz *et al.*, 2010). However, HoxH_F37 and HoxH_R518 have substantially fewer primer-template mismatches to publicly available bidirectional [NiFe] hydrogenase genes than the bidirectional [NiFe] hydrogenase-specific primer pair HoxHf and HoxHr (Table S4). Of the 39 publicly available *Cyanobacteria* or *Chloroflexales* bidirectional [NiFe] hydrogenases 38 had ≤ 2 mismatches to HoxH_F37/HoxH_R518 compared to 9 of 39 for HoxHf/HoxHr, indicating that HoxH_F37/HoxH_R518 targets a larger portion of bidirectional [NiFe] hydrogenases from *Cyanobacteria* and *Chloroflexales*. Analysis of a selection of uptake [NiFe] hydrogenases showed that all had ≥ 3 primer-template mismatches to HoxH_F37/HoxH_R518 (Table S4). Amplicons of the expected size were generated from a selection of *Cyanobacteria* strains and one *Chloroflexales* strain that have a bidirectional [NiFe] hydrogenase, but these amplicons were not generated from *Crocospaera watsonii* WH 8501, a cyanobacterium that does not have a bidirectional [Nife] hydrogenase but has an uptake [NiFe] hydrogenase (Table S5).

A total of 205 bidirectional [NiFe] hydrogenase gene fragments were amplified from DNA (91 clones) and RNA (cDNA; 114 clones) templates obtained from the upper photosynthetic layer (0-2 mm) of Elkhorn Slough mats under dark anoxic conditions at midnight, Nov 11, 2009. The gene fragments formed 9 distinct clusters (including 5 singleton clusters) at the 97% similarity threshold (Table 4; Figure 3). The majority of the sequences were affiliated with *M. chthonoplastes* PCC7420 (HoxH1 and HoxH2 clusters). The HoxH1 cluster represented 31% of DNA clones and 61% of cDNA clones (98% identity; $>65\%$ bootstrap support for branch point) and the HoxH2 cluster represented 3% of DNA clones and 15% of cDNA clones (96% identity; $>50\%$ bootstrap support for branch point). Another highly represented cluster, HoxH4 (48% of DNA

clones; 9% of cDNA clones), was most closely related to *Cyanothece* sp. PCC8802 (70% identity; unresolved branch point). Clusters HoxH1 and HoxH2 were relatively active, with a cDNA:DNA >1, whereas HoxH4 was relatively inactive with a cDNA:DNA <1.

Discussion

Analyses of rRNA and H₂-evolving bidirectional [NiFe] hydrogenase genes and transcripts combined with biogeochemical measurements across a range of manipulations strongly suggest that fermentation of stored photosynthate by *Microcoleus* spp. is a major source of H₂ in Elkhorn Slough mats.

H₂ production occurred predominantly in the upper photosynthetic layer (0-2 mm) of Elkhorn Slough mats. Similar results have been reported in other photosynthetic mats from Guerrero Negro, Mexico (Hoehler *et al.*, 2001), suggesting that quantitatively important hydrogenogens are active in the upper layer of geographically diverse mats. Elkhorn Slough mats also harbored SRB (*Desulfobacterales*; Table 3), a group known to be actively reducing sulfate under oxic day conditions and anoxic nighttime conditions in photosynthetic microbial mats (Canfield and Des Marais, 1991). These microorganisms include hydrogenotrophs (Widdel, 1987) and thus H₂ production observed in our experiments is likely a measurement of net H₂ production. Our observations cannot rule out that enhanced H₂ consumption in deeper layers accounts for the lower net H₂ production relative to the upper photosynthetic layer. Indeed, we anticipate that more stable physico-chemical conditions within the permanently dark and anoxic portion of the mat should allow for the development of closer associations between hydrogenogens and hydrogenotrophs and thus a lower net efflux of H₂ than in the highly dynamic upper

phototrophic layer. However, we hypothesize that the observed rates of H₂ and organic acid production are unlikely to be sustained in lower aphotic layers, which lack daily photosynthetic replenishment of the fixed carbon pool and therefore have a discrete reservoir of carbon that would be rapidly depleted. Further investigation of hydrogenotrophs is needed to better understand H₂ ecology and the H₂ producing capacity of microbial mats.

Previous research of microbial mats from Shark Bay, Australia had concluded that co-metabolic production of H₂ during N₂-fixation could be an important contributor to net H₂ production as N₂-fixation was observed to occur contemporaneously with H₂ production (Skyring *et al.*, 1989). Similar patterns of N₂-fixation and H₂ production were observed in mats from Elkhorn Slough. However, further examination of the role of N₂-fixation in H₂ production using N₂-fixation suppression experiments indicated that H₂-production is independent of N₂-fixation and most likely fermentative due to the simultaneous production of organic acids (expected fermentation products) under dark, anoxic conditions.

Inhibition of oxygenic and/or anoxygenic phototrophic activity by depriving the microbial mats of solar irradiance or by addition of DCMU suggested that phototrophs were important contributors to the observed H₂ production observed during the subsequent nighttime period. However, biogeochemical data alone did not provide evidence to identify the type of phototroph or the taxonomic identity of microorganisms producing H₂.

399 Our analysis of rRNA genes and transcripts demonstrated that *Cyanobacteria*,
400 specifically *Microcoleus* spp. were important members of the active microbial
401 community. We obtained similar results to an investigation of photosynthetic mats from
402 Guerrero Negro, Mexico by Ley and colleagues (2006). In both studies, *Cyanobacteria*
403 dominated the active microbial communities as determined by rRNA transcript analysis
404 despite being substantially less significant within the total (active + inactive) community
405 as determined by rRNA gene analysis. These observations underscore the need to
406 investigate both the presence and activity of microorganisms in complex microbial
407 ecosystems to gain a better understanding of their likely metabolic importance.

408 Some cyanobacterial strains isolated in pure culture from microbial mats are capable of
409 fermentative H₂ production from stored photosynthate, including glycogen (Stal and
410 Moezelaar, 1997). No studies have been carried out on true *Microcoleus* sp. isolates to
411 ascertain their capabilities of producing H₂ (Bolhuis *et al.*, 2010; Garcia-Pichel *et al.*,
412 1996; Moezelaar *et al.*, 1996). However, recent analysis of publicly available
413 cyanobacterial genomes have shown that all *Cyanobacteria* (including *M. chthonoplastes*
414 PCC7420) harboring a bidirectional [NiFe] hydrogenase also harbor a
415 pyruvate:flavodoxin/ferredoxin oxidoreductase (PFOR), leading to speculation that the
416 bidirectional [NiFe] hydrogenase is used to shunt electrons (as H₂) via a PFOR-like
417 enzyme during fermentation (Barz *et al.*, 2010). H₂-evolving bidirectional [NiFe]
418 hydrogenase clone libraries constructed from the upper photosynthetic layer of Elkhorn
419 Slough mats using degenerate primers designed to equally target many *Cyanobacteria*
420 and *Chloroflexales* were dominated by sequence clusters most closely affiliated with *M.*
421 *chthonoplastes* PCC7420 (Figure 3). Overrepresentation of hydrogenases in the cDNA

library for clusters most closely affiliated with *M. chthonoplastes* PCC7420 (Table 4) provides further evidence that *Microcoleus* spp. are the main hydrogenogens in Elkhorn Slough mats.

Data obtained in this study indicated that anoxygenic phototrophy can drive H₂ production in microbial mats. Facultative anoxygenic phototrophy in *Cyanobacteria*, including *Microcoleus* sp. has been reported (Cohen *et al.*, 1986; De Wit *et al.*, 1988; Garlick *et al.*, 1977). However, misidentification of cyanobacterial strains as *Microcoleus* sp., including ‘strain 11’ used by de Wit and colleagues (1988) has been documented (Bolhuis *et al.*, 2010; Garcia-Pichel *et al.*, 1996) and thus it is unclear if *Microcoleus* spp. are capable of anoxygenic phototrophy. The capability of *Microcoleus* spp. to switch between oxygenic and anoxygenic phototrophy might be important to their survival and proliferation in microbial mats where sulphurous compounds can be prevalent and inhibit PSII activity (Jorgensen *et al.*, 1986). Alternatively, anoxygenic phototrophic *Chloroflexi* strains may have a role in H₂ production. Analysis of nighttime rRNA gene and rRNA transcript libraries revealed that OTU 31 affiliated with *Chloroflexales* and OTU 1176 affiliated with *Chloroflexi* were overrepresented in the rRNA transcript libraries having %rRNA transcript:rRNA gene ratios >1 in both nighttime samples (Table 3), indicating that they were consistently relatively active when H₂ production was pronounced. Cloning of H₂-evolving bidirectional [NiFe] hydrogenases did not indicate that *Chloroflexales* bidirectional [NiFe] hydrogenases were expressed. It should be noted that although degenerate primers were designed to amplify bidirectional [NiFe] hydrogenases from a diversity of *Cyanobacteria* and *Chloroflexales*, a relatively high number of mismatches (≥2) between both forward and reverse primers

to the bidirectional [NiFe] hydrogenase gene was determined for one of the *Chloroflexales* members, *Oscillochloris trichoides* DG6 (Table S4). Thus, it is possible that *Oscillochloris*-like spp. or other as yet unknown *Chloroflexales* may contribute to the observed H₂ produced in Elkhorn Slough mats. This is further supported by the observation that the closest pure culture strain match to OTU 31 and 1176, OTUs determined to be relatively active under H₂-producing conditions based on rRNA gene and rRNA transcript pyrosequencing, was *Oscillochloris* sp. BM (Table 3).

While we do not rule out a role for *Chloroflexales* in H₂ production, analysis of pyrotags and [NiFe] hydrogenases indicates that *Cyanobacteria*, including *Microcoleus* spp., were relatively more active than *Chloroflexales*. Also, the dominance of *Microcoleus* spp. within the active fraction of the microbial community in the H₂ producing layer suggests that the contribution of microorganisms other than *Cyanobacteria* to H₂ production is likely minimal. Together, these biogeochemical data, manipulations and molecular data demonstrate that *Microcoleus* spp. are dominant hydrogenogens in Elkhorn Slough mats.

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Table 1. Sampling times and statistics of rRNA gene and transcript pyrosequencing.

Library	Time and date	Pool	Filtered reads (+) singletons	Filtered reads (-) singletons	OTUs (97%) (+) singletons	OTUs (97%) (-) singletons
#1	Day, 12:00 11/10/2009	DNA	5796	5576	737	380
#2	Day, 12:00 11/10/2009	RNA	5756	5641	486	233
#3	Night, 0:00 11/11/2009	DNA	1191	1147	291	125
#4	Night, 0:00 11/11/2009	RNA	5310	5230	357	166
#5	Day, 12:00 11/12/2009	DNA	4487	4402	384	194
#6	Day, 12:00 11/12/2009	RNA	4825	4780	242	121
#7	Night, 0:00 11/13/2009	DNA	5573	5234	866	390
#8	Night, 0:00 11/13/2009	RNA	4091	4038	275	121

Table 2. Richness and diversity estimates of OTUs (97% similarity).

Library	OTU richness ^a			OTU diversity ^b	
	Observed	Chao1	ACE	Simpson ^c	Shannon
#1 (Day 1 ^c , DNA)	737	1309 (±86)	1334 (±22)	52.84	5.08
#2 (Day 1, RNA)	486	880 (±70)	952 (±19)	5.30	3.15
#3 (Night 1 ^c , DNA)	291	633 (±81)	616 (±16)	29.55	4.52
#4 (Night 1, RNA)	357	645 (±59)	681 (±15)	3.88	2.63
#5 (Day 2 ^c , DNA)	384	723 (±72)	715 (±16)	14.10	3.77
#6 (Day 2, RNA)	242	469 (±62)	433 (±13)	3.38	2.18
#7 (Night 2 ^c , DNA)	866	1679 (±107)	1813 (±28)	35.20	4.96
#8 (Night 2, RNA)	275	543 (±63)	613 (±16)	3.93	2.50

^aChao1 and ACE (abundance based coverage estimators) indices are non-parametric estimators of species (OTU) richness (total number of different OTUs in a given sample).

^bShannon and Simpson diversity indices consider both species richness and evenness (relative abundance).

^cDay 1 is 12:00, November 10, 2009; Night 1 is 0:00, November 11, 2009; Day 2 is 12:00, November 12, 2009; Night 2 is 0:00, November 13, 2009.

^dInverse simpson values are reported.

Table 3. %rRNA transcript :%rRNA gene ratios of the most relatively active OTUs observed in all nighttime pyrotag libraries

OTU ^a	Most related sequences ^b	Accession number	Identities ^c	E-value ^d	OTU taxonomic affiliation ^e	Ratio ^f Night 1 Nov/11/2009	Ratio ^f Night 2 Nov/13/2009
2	<i>Microcoleus chthonoplastes</i>	AM709630	200/200	6e-97	<i>Microcoleus</i> spp.	6.0	4.2
	<i>Microcoleus chthonoplastes</i>	GQ402023	200/200	6e-97			
225	<i>Desulfococcus</i> sp.	EF442980	194/200	9e-89	<i>Desulfobacterales</i>	5.5	2.6
	<i>Desulfococcus multivorans</i>	AF418173	194/200	9e-89			
5	<i>Leptolyngbya fragilis</i>	HQ832925	198/200	3e-94	<i>Oscillatoriales</i>	4.1	3.2
	Coral bacterium clone	FJ203604	193/200	4e-87			
9	Mat bacterium clone	EU246323	200/200	7e-97	<i>Cyanobacteria</i>	3.3	3.3
	<i>Phormidium</i> cf. <i>formosum</i> ^b	EU196640	190/200	2e-83			
31	Mat chloroflexales clone	AJ308496	200/200	7e-97	<i>Chloroflexales</i>	4.8	1.5
	<i>Oscillochloris</i> sp. ^b	AF149018	188/200	2e-78			
387	Microbialite nematoda clone	GQ483712	200/200	7e-97	<i>Nematoda</i>	2.1	3.7
	<i>Eumonhystera</i> cf. <i>simplex</i> ^b	AY284692	177/200	6e-66			
379	Wetland cytophagales clone	FJ516772	188/200	1e-80	<i>Bacteroidetes</i>	1.6	3.8
	<i>Aquiflexum balticum</i> ^b	NR025634	186/200	7e-78			
1176	Mat chloroflexi clone	DQ329977	199/200	3e-95	<i>Chloroflexi</i>	1.9	2.9
	<i>Oscillochloris</i> sp. ^b	AF149018	178/200	5e-67			

^aOTU identification number is arbitrary and was designated by PyroTagger.

^bThe NCBI non-redundant nucleotide sequence database was queried to identify closest matches to OTU sequences; closest pure culture match where a given OTU has its closest matches to only uncultured clone sequences.

^cBLASTN uses an identity matrix for nucleotide comparisons, see <http://www.ncbi.nlm.nih.gov/BLAST/>; total length of query was 200 bp.

^dE-value or expect value describes the significance of the match between two sequences, see <http://www.ncbi.nlm.nih.gov/BLAST/>.

^eFor OTU taxonomic affiliation designation see methods (*Pyrosequencing of SSU rRNA genes and rRNA transcripts*).

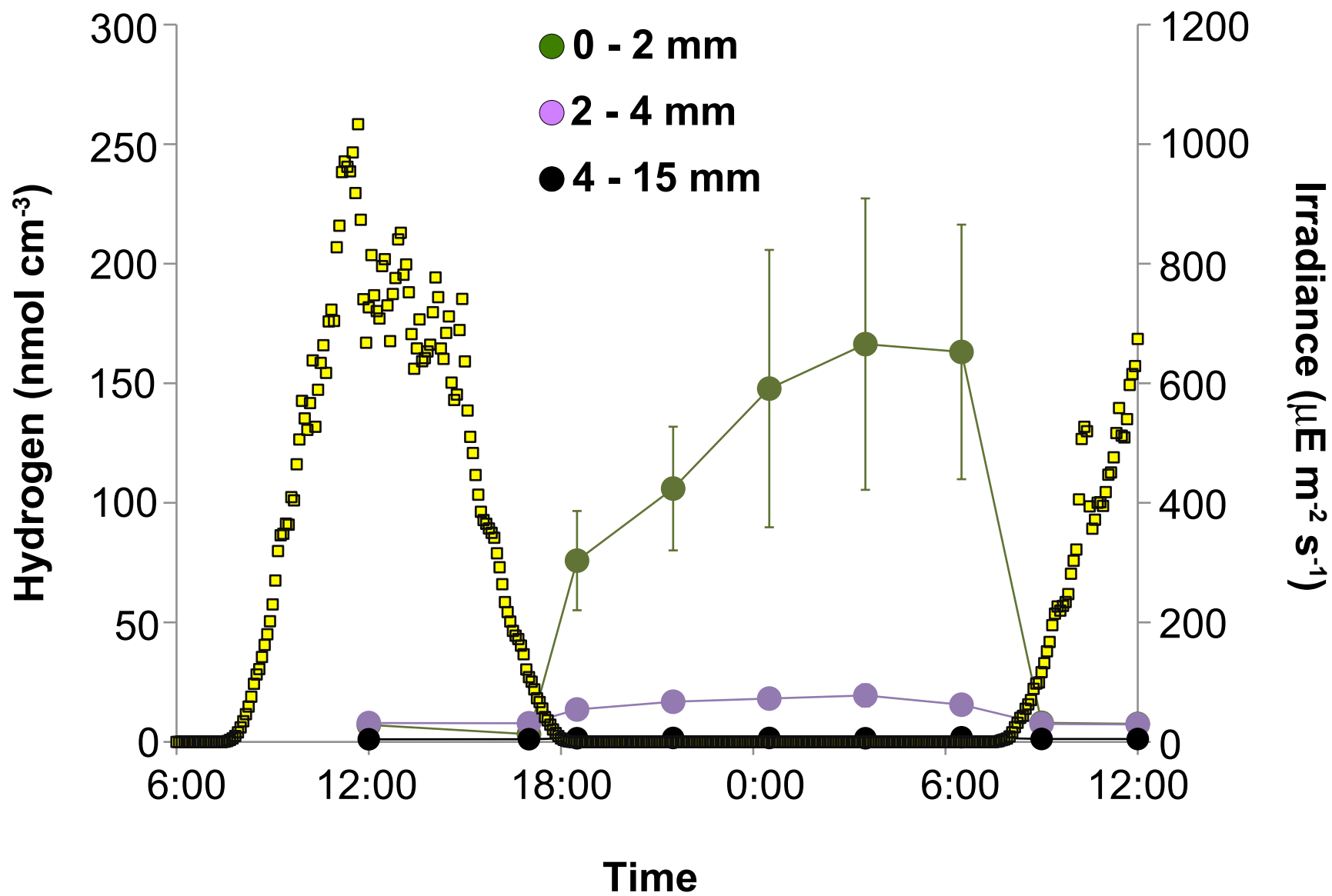
^frRNA transcript:rRNA gene ratios were calculated for sequences recovered from DNA or cDNA template based libraries constructed from Night 1 (0:00) November 11, 2009 or Night 2 (0:00) November 13, 2009 samples by dividing the percentage abundance of pyrosequence tags in the rRNA transcript library by the percentage abundance in the rRNA gene library.

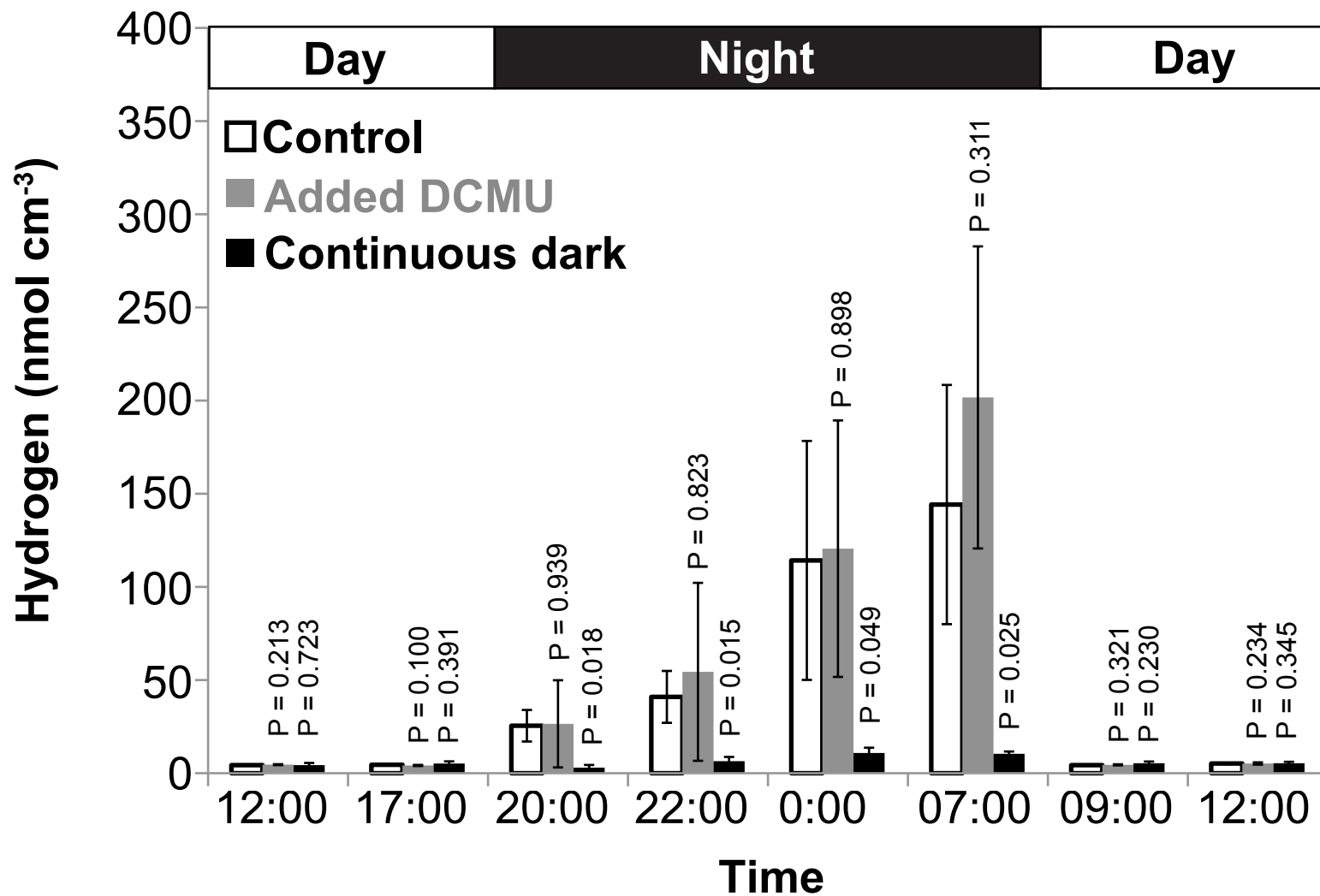
Figure legends

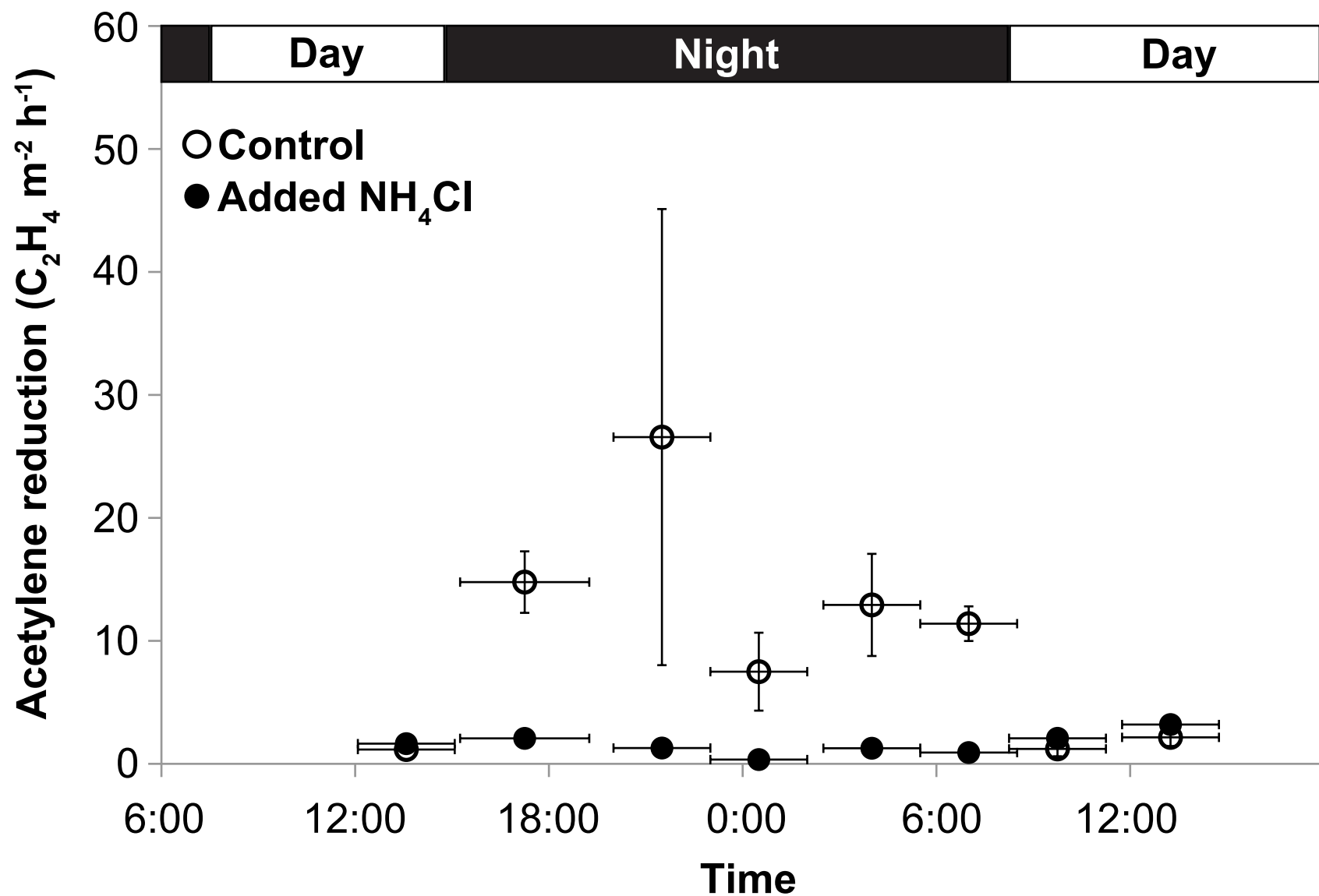
Figure 1. Diel production of H₂ or organic acids under manipulated conditions in Elkhorn Slough mats. Mat cores incubations in vials commenced at dawn with H₂ and organic acids allowed to accumulate over a diel cycle with N₂ flushing of the vial at the end of the day and night periods. Vials in which organic acids were measured were reset at dawn by replacement with fresh field site water (amended as appropriate for manipulated conditions). Vertical error bars indicate the standard deviation of six replicates (H₂) or three replicates (organic acids). Asterisks denote significant differences between control and manipulated conditions (P values < 0.05). **(a)** H₂ production in different layers of microbial mat. Mat cores were sectioned at different depths, 0-2 mm, 2-4 mm and 4-15 mm. Yellow squares denote natural solar irradiance. μE , microeinsteins. **(b)** H₂ production under control, DCMU (20 μM) added and continuous dark conditions. **(c)** H₂ production under control and NH₄Cl (8.8 mM) added conditions. **(d)** Organic acid production under control and NH₄Cl (8.8 mM) added conditions.

Figure 2. Ranked barplot of the six most abundant phyla of the rRNA gene (left) and rRNA transcript (right) libraries. Day 1 is 12:00 November 10, 2009, Day 2 is 12:00 November 11, 2009, Night 1 is 0:00 November 11, 2009 and Night 2 is 0:00 November 13, 2009. OTUs within the same phyla are stacked in order of relative abundance and separated graphically into segments. The most abundant OTUs (#2; *Microcoleus* spp. and #9; *Cyanobacteria*) are labeled according to their (arbitrary) OTU identification number assigned during PyroTagger clustering.

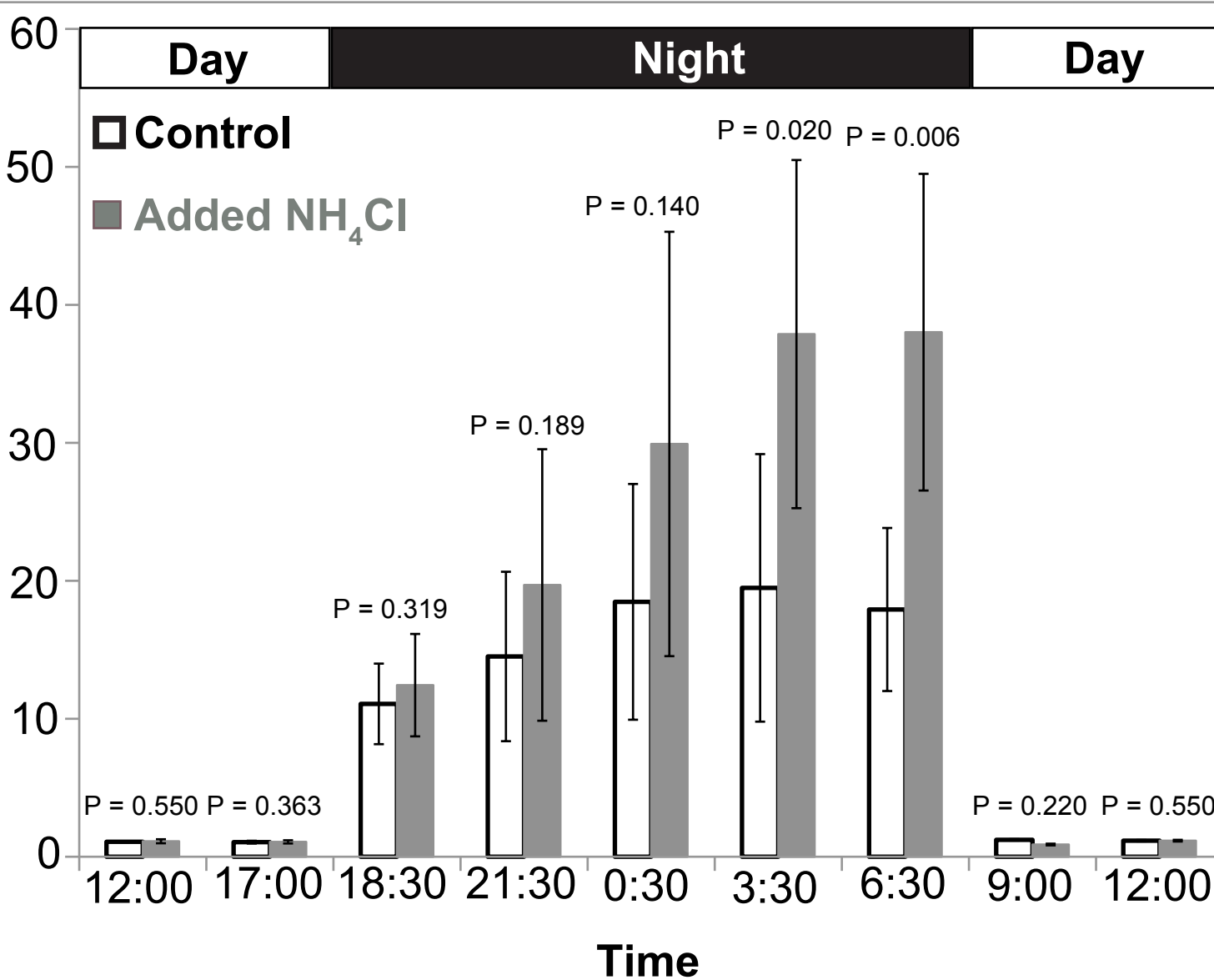
Figure 3. Maximum-likelihood phylogenetic tree of bidirectional [NiFe] hydrogenases. Branch points supported by all phylogenetic analyses (maximum likelihood using Dayhoff or WAG amino acid correction and maximum parsimony evolutionary models) and their bootstrap support are denoted. The bar represents 0.1 changes per amino acid and the outgroup was *Methanococcus voltae* A3 HoxH.



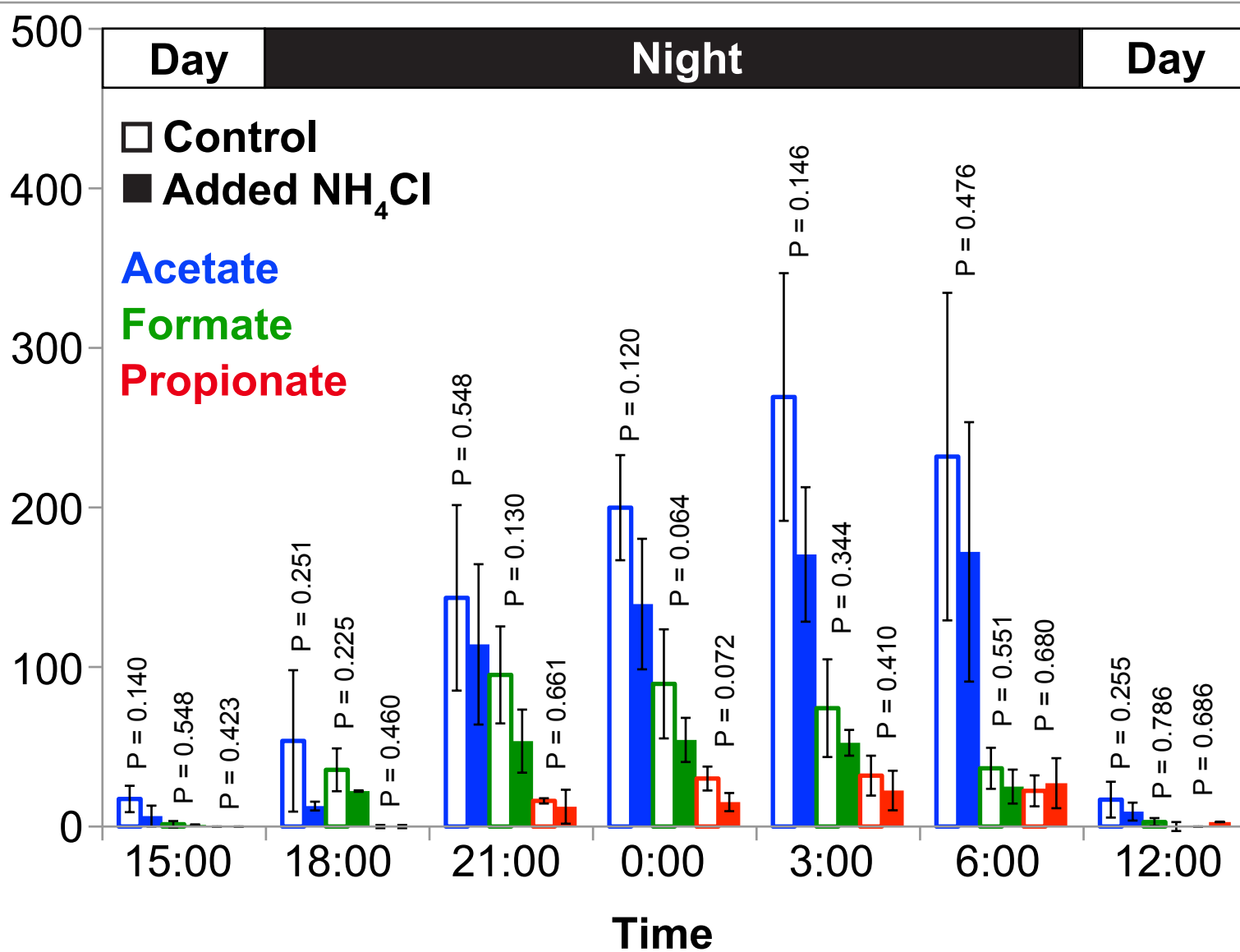


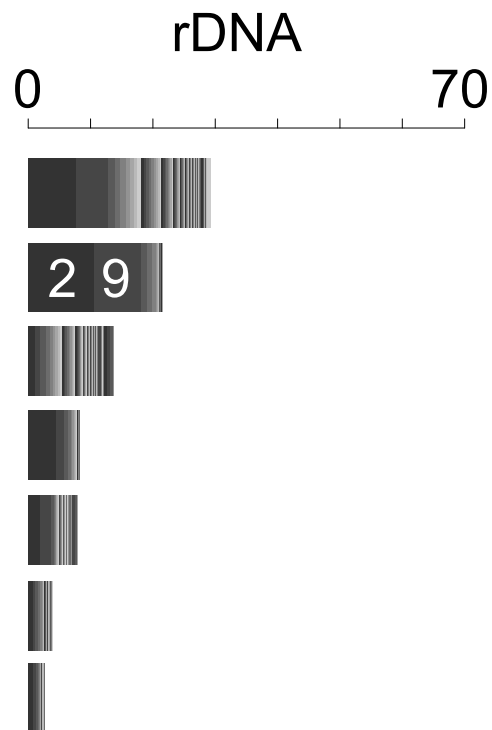
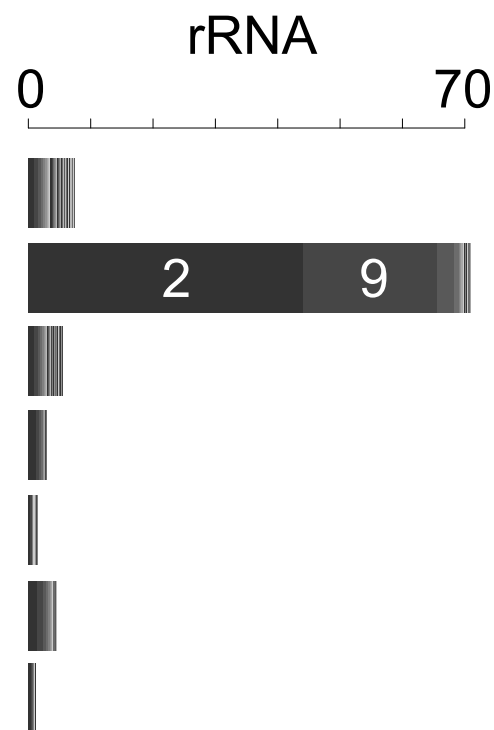


Hydrogen (nmol cm⁻³)



Organic acids (nmol cm⁻³)



A**% Abundance****B**

- >95% bootstrap support
- >80% bootstrap support
- >65% bootstrap support
- >50% bootstrap support

